Acta Biomaterialia 25 (2015) 230–239

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Establishing correlations in the en-mass migration of dermal fibroblasts on oriented fibrillar scaffolds

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ARTICLE INFO

Article history:
Received 25 January 2015
Received in revised form 14 June 2015
Accepted 23 June 2015
Available online 24 June 2015

Keywords:
Three dimensional scaffolds
Dermal fibroblasts
Cell migration
Oriented fibers

ABSTRACT

Wound healing proceeds via fibroblast migration along three dimensional fibrillar substrates with multiple angles between fibers. We have developed a technique for preparation of three dimensional fibrillar scaffolds with where the fiber diameters and the angles between adjacent fiber layers could be precisely controlled. Using the agarose droplet method we were able to make accurate determinations of the dependence of the migration speed, focal adhesion distribution, and nuclear deformation on the fiber diameter, fiber spacing, and angle between adjacent fiber layers. We found that on oriented single fiber layers, whose diameters exceeded 1 μm, large focal adhesion complexes formed in a linear arrangement along the fiber axis and cell motion was highly correlated. On multi layered scaffolds most of the focal adhesion sites reformed at the junction points and the migration speed was determined by the angle between adjacent fiber layers, which followed a parabolic function with a minimum at 30°. On these surfaces we observed a 25% increase in the number of focal adhesion points and a similar decrease in the degree of nuclear deformation, both phenomena associated with decreased mobility. These results underscore the importance of substrate morphology on the en-mass migration dynamics.

Statement of Significance

En-mass fibroblast migration is an essential component of the wound healing process which can determine rate and scar formation. Yet, most publications on this topic have focused on single cell functions. Here we describe a new apparatus where we designed three dimensional fibrillar scaffolds with well controlled angles between junction points and highly oriented fiber geometries. We show that the motion of fibroblasts undergoing en-mass migration on these scaffolds can be controlled by the substrate topography. Significant differences in cell morphology and focal adhesions was found to exist between cells migrating on flat versus fibrillar scaffolds where the migration speed was found to be a function of the angle between fibers, the fiber diameter, and the distance between fibers.

1. Introduction

Collective migration where cells migrate together while maintaining cell–cell contacts has been studied extensively especially for epithelial and endothelial cells, which migrate as a sheet [1–5]. Other studies have focused on migration of multiple cell types together in order to determine the role of soluble factors in the process [6,7]. In vivo wound healing is a combination of multiple factors, where the rate of the migration process can be a determinant factor in controlling the probable outcome-i.e. low mobility can prevent healing as has been reported for diabetics while overly rapid rates are associated with hypertrophic scar formation [8–10]. The rapid developments in the stem cell field has amply demonstrated that the mechanical, chemical, and morphological properties of the substrates on which the stem cells reside, both in vivo and in vitro, can be determining factors [11–14]. Since wound healing also involves tissue regeneration and renewal, these factors need to be considered as well. Fibroblasts, which play a major role in collagen contraction, are particularly sensitive to environmental cues such as the diameter of the collagen fibers [15] or their rigidity, where both factors were shown in vitro and in vivo to influence cell migration and healing [16,17].

Even though fibroblasts do not migrate as a sheet, it was shown that single cell migration can differ fundamentally from the

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http://dx.doi.org/10.1016/j.actbio.2015.06.030
1742-7061/

migration which occurs when multiple cells are involved. This type, referred to as "en-mass" migration [18,19], relies on cell–cell communication and interactions via the substrate properties, where studies that have shown that fibroblasts prefer fibrillar structure over than flat surfaces [20–23].

Yet despite its importance, most studies have focused on the dynamics of isolated cells, [24–27] and only few studies have been reported regarding en-mass migration [19,28]. For example, Liu et al. [28] compared en-mass migration of fibroblasts out of an agarose droplet to single cell migration onto flat and fibrillar surfaces. On flat surfaces, fibroblasts migrated outward into a "sunburst" type pattern in a trajectory aimed increasing the separation between adjacent cells while the speed of the cells decreased with increasing distance from the droplet, reaching the single cell migration value after 24 h. In contrast, cells migrating outward from droplets placed on fibers experienced a "bottleneck" effect, as they emerged en-mass with the single cell velocity which remained unchanged for the first 24 h. It has also been shown that three dimensional migration of human dermal fibroblasts on a fibrous scaffolds, having diameters 1 μm or larger, did not proceed via cell migration within the pores of the scaffold [29]. Rather, the migration occurred only along the fibers, where motion from one layer to the next involved a sudden change in trajectory at the junction of fibers in different layers. Hence it appeared that the major factors determining the magnitude and direction of the migration speed were the nature of the fiber junctions, rather than the porous structure of the scaffold.

It has been shown in several studies that cell migration was affected by the topography of the underlying substrate [20,30–35], which in turn determined other parameters such as density and distribution of the focal adhesion points [36–40] the degree of nuclear deformation [41–44]. In the case of en-mass migration of cells on scaffolds, the angle between fibers, or the angle at the junction points, can also be element determining the substrate topography. Hence the question arises whether cells could discriminate between junction points having different angles and exhibit a preference for migration in a specific direction.

In order to address these questions, we have devised a technique for producing a multi-layered scaffold where the angle between fibers can be precisely controlled and the influence of the three dimensional substrate morphology on the migration behavior determined. We chose to use the well-established droplet technique [45–47] for studying en-mass migration behavior rather than scratch assays [48–50] since it allows for better quantification of the cell migration trajectories and the behavior of individual cells within the migrating ensemble. The answers to these questions can further our understanding of the elements within the substrate that control outward cell migration and can enhance our ability to engineer structures essential for wound healing applications.

2. Materials and methods

2.1. Fabrication of electrospun PMMA scaffolds of variable angle

The application of the multilayered scaffold is preceded by preparation of the substrate with a flat spun cast film. The film allows for adhesion of the scaffold, as well as for a direct comparison between cells on flat vs fibrillar surfaces. Poly (methyl methacrylate) (PMMA) (Mw = 120,000 Da, Mw/Mn = 3; Sigma–Aldrich) was dissolved in toluene (Fisher Scientific, Pittsburgh, PA) at 30 mg/mL, and then spin-casted on 1.5 cm diameter glass cover slips at 2500 RPM for 30 s.

Electrospun fibers of different diameters were produced by dissolving PMMA in different solvents, as listed in Table 1, where the solvent and concentrations were optimized to minimize beading and produce fibers of the desired diameters.

Table 1
Solvent and concentration of PMMA for producing different sizes of electrospun fibers.

Concentration of PMMA (%)	Solvent	Results
30	Tetrahydrofuran + Dimethylformamide (1:1)	1 μm fibers
30	Dimethylformamide + Chloroform (1:1)	4 μm fibers
20	Chloroform	8 μm fibers

The set up for obtaining oriented multi layered scaffolds is illustrated in Fig. 1a. Fiber alignment was accomplished by placing the PMMA coated cover slips on a drum whose diameter, 10 cm, was much larger than that of the cover slips, and which was rotating at a speed of 6750 RPM. Analysis of multiple images and more than 500 fibers indicated that the degree of alignment, as determined from the ratio of aligned vs. curved, non-aligned fibers was greater than 98%. The distance between adjacent fibers was controlled by the electrospinning time, where mean distances of 30, 50, and 100 μm were achieved with spinning times of 1, 2.5, and 5 min. The angular orientation of the cover slips was marked by fiduciary on the drum as shown. The angle between different layers could then be precisely controlled via rotation of the cover slip after deposition of each layer. The samples were then annealed in a vacuum of 10^{-7} Torr at 120 °C overnight to remove the remaining solvent and sterilize the samples.

Fig. 1b–f shows the phase contrast images of samples with angles of 15°, 30°, 45°, 75°, and 90° between adjacent layers of fibers. From the figure we can see that even though the spacing between fibers has a large variation, the angle between fibers in adjacent layers is precise to within 5°. We have previously shown that for an orthogonal scaffold, the spacing between fibers need only be large enough to allow cells attached to fibers to penetrate, and hence did not affect the migration when they were in a range from 10 to 100 μm. The spacing between the fibers shown in Fig. 1b–f was easily controlled within this range and hence no further processing [51,52] was applied to try to reduce the variation.

2.2. Cell culture

Adult Human Fibroblast Cell (CF29, passage 10-passage 12 cells were used in this study) was purchased from ATCC (Manassas, VA), and cultured in Dulbecco's Modified Eagle Medium (DMEM), together with 10% fetal bovine serum (Hyclone, Logan, UT), 1% antibiotic mix of penicillin, streptomycin, and L-glutamine (GIBCO BRL/Life Technologies, Grand Island, NY), (referred to as full-DMEM), in a humidified incubator at 37 °C with 5% CO_2. Before seeding the cells, the samples were further sterilized by exposure to the antimicrobial UVC light within the BSL-2 enclosure for 20 min, and then submerged in 0.5 ml of 30 mg/mL of intact human plasma fibronectin (Calbiochem, San Diego, CA) in serum-free DMEM for 2 h in the incubator. Sterilization with alcohol was avoided since it pitted the PMMA surfaces.

2.3. Cell membrane staining

Fibroblasts cell membranes were live stained with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD, Invitrogen, Carlsbad, CA). Cells were washed with phosphate buffer saline (PBS), and suspended in the 3.5 μg/ml DiD-serum free